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TITLE: THE PRIMARY SEQUENCE OF ACETYLCHOLINESTERASE AND
          SELECTIVE ANTIBODIES FOR THE DETECTION OF
          ORGANOPHOSPHATE TOXICITY

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Acetylcholinesterase polymorphism is a consequence of alternative mRNA processing which gives rise to two distinct molecular species of acetylcholinesterase. The work summarized in the progress report characterized the protein structure of acetylcholinesterase from Torpedo and correlated it with the organization of the gene. A divergence is found at the carboxyl-terminus of the molecule giving rise to a hydrophilic, oligomeric and a glycophospholipid-linked species. Antibodies which react uniquely with each form of the enzyme have been prepared which revealed each enzyme species to be localized at distinct positions with the synapse. Other antibodies defined a unique carbohydrate epitope on the glycophospholipid-linked form and revealed that the active center of the enzyme is occluded from the surface. Distinct epitopes within the active center of the enzyme were also delineated. Lastly, we have developed an expression system using a Spodoptera-baculovirus system which has enabled us to express recombinant derived enzymes in large quantity. The enzyme can be purified in good yield and to date Torpedo wild-type, Torpedo mutants and mouse recombinant enzymes have been expressed in this system. Human recombinant AChE has also been expressed in a transient transfection system.

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FOREWORD

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The Primary Sequence of Acetylcholinesterase
and Selective Antibodies for the Detection of
Organophosphate Toxicity

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I. INTRODUCTION

Sir Henry Dale, in 1914, suggested that an enzyme which degrades the esters of choline played a role in neurotransmission within the autonomic and somatic motor nervous system and that this enzyme, acetylcholinesterase (AChE), was the target of the action of the drug, physostigmine[1]. Since the mid-19th century[2], AChE inhibitors have enjoyed therapeutic applications in the treatment of glaucoma, smooth muscle atony, certain arrhythmias, and myasthenia gravis, and have been used to reverse competitive neuromuscular blockade[3]. Recently, reports of beneficial actions of long acting AChE inhibitors in the amelioration of Alzheimer's disease have appeared[4]. In addition, these agents have been used widely as agricultural and garden insecticides. Early studies also established that AChE catalysis was typical of serine hydrolases. The pioneering work of Irwin Wilson and his colleagues demonstrated the principle of site direction for developing both selective inhibitors and reactivators of the enzyme[5].

A critical direction in the study of the structure of the AChE's was set with the finding of Massoulié and Reiger[6] that a synaptic form of AChE was dimensionally asymmetric and linked to a filamentous structural subunit. Treatment of these species with proteases or collagenases removed the tail unit without loss of catalytic activity and resulted in a globular tetramer of subunits. It was later established that the tail unit contained a collagen-like composition[7,8]. The diversity of molecular species of AChE has become more complex and it is now appropriate to divide them into two classes (fig. 1).

Fig. 1 Polymorphic forms of AChE can be divided into two classes: (1) heteromeric associations of catalytic and structural subunits (Included in this group are dimensionally asymmetric forms which contain multiple catalytic subunits disulfide-linked either to a collagen-like tail unit or to a lipid-linked subunit), and (2) homomeric forms, which contain associations of identical subunits and differ in hydrophobicity by attachment of a glycophospholipid.

The heteromeric class contains catalytic subunits disulfide-linked to structural subunits. It includes the asymmetric, collagen-containing species (designated 'A' forms) found largely in the neuromuscular junction and ganglia[7,8] and a second species, found in brain, in which a tetramer of catalytic subunits is disulfide-bonded to a lipid-linked subunit[9].
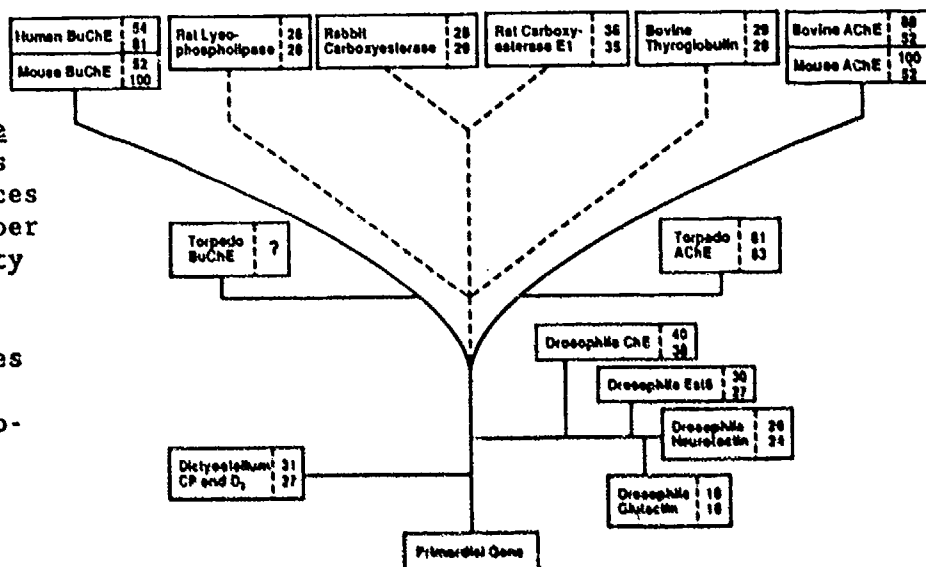
The homomeric forms consist of species with an association of identical subunits. Those that are membrane-associated contain a glycophospholipid moiety covalently linked to the C-terminal carboxyl residue in the protein [designated 'H' (for hydrophobic) forms][10,11]. Hence, the membrane-associated

1

forms have distinctive means for tethering themselves to the outside surface
of the cell. The expression of a particular molecular species of AChE is
under the control of phenomena related to cellular excitability, such as
intracellular $[Ca^{++}]$, synaptogenesis, and the formation of action potentials.

Enzyme Structure

AChE was first cloned in 1986 from Torpedo[12]. Since that time sequence of
Drosophila cholinesterase, human, rabbit and mouse butyrylcholinesterase
(BuChE) and mouse, human and bovine AChE have been reported[13-18]. The
cholinesterase sequence defined a new family of serine hydrolases distinct
from the pancreatic and Subtilisin families of hydrolases[12] (fig. 2).

Fig. 2 Cholinesterase Gene
Family. Sequence identities
come from published sequences
(refs. 12-24). The top number
reflects amino acid identity
with murine AChE and the
bottom number with murine
BuChE. Lengths of the lines
are arbitrary and do not
represent a statistical evo-
lutionary tree.

Included with this functionally eclectic family are thyroglobulin, Drosophila
neurotactin and glutactin, a Dictyostelium esterase of unknown function, and
esterases from the male reproductive organ of Drosophila and from the
endoplasmic reticulum of mammalian liver. Moreover, all of the
cholinesterases maintain close sequence identity. Analysis of the disulfide
bonding pattern in AChE reveals that of the eight cysteines, six are conserved
in the three intrasubunit linkages and one (C-231) is free, while C-572 is
involved in intersubunit disulfide bonding[25]. That the six cysteines involved
in intrasubunit bonding are all conserved in the large gene family indicates
that all members have identical folding patterns. The inferred cDNA sequence
also reveals a 21-amino-acid-leader peptide but no other obvious membrane
spanning regions. Hence, the encoded sequence is targeted for secretion from
the cell, and post-translational modifications are responsible for the
membrane associations seen in situ.

The core AChE sequence is common to all of the homologous proteins; in
turn, they may have divergent amino-terminal and carboxyl-terminal sequences.
Hence, a common sequence has served nature as a scaffold upon which esterase
active sites, precursor tyrosines for thyroid hormone (thyroglobulin) and
heterologous cell contact molecules have been added (fig. 3). Moreover, the
diversity within the cholinesterases arises in the very carboxy-terminal end
of the cholinesterases. These divergent domains are responsible for
determining the cellular disposition of the molecule.

DICTYOSTELIUM CRYSTAL PROTEIN
DICTYOSTELIUM D2 ESTERASE
DROSOPHILA NEUROTACTIN
DROSOPHILA GLUCTACTIN
JUVENILE HORMONE ESTERASE
DROSOPHILA ESTERASE 6
DROSOPHILA ESTERASE P
DROSOPHILA AcChE
TORPEDO AcChE
MOUSE, BOVINE and HUMAN AcChE
MOUSE, RABBIT and HUMAN BuChE
RAT ESTERASE
RABBIT ESTERASE 1
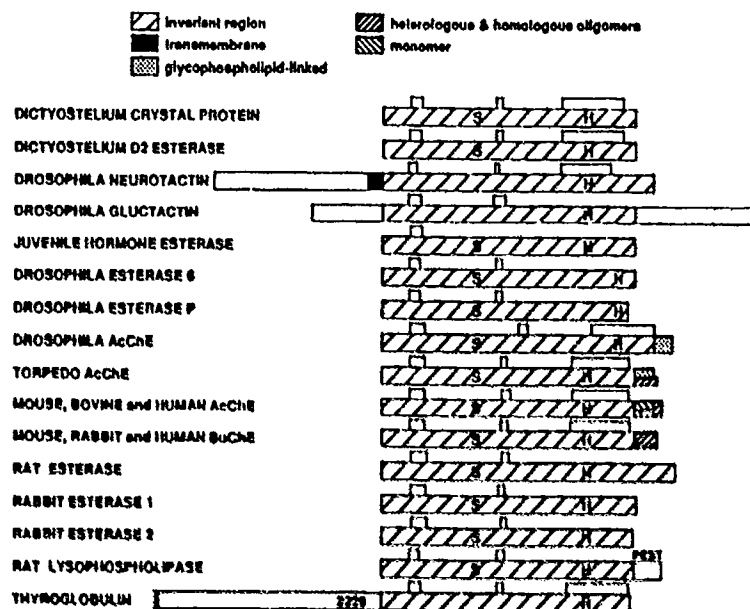RABBIT ESTERASE 2
RAT LYSOPHOSPHOLIPASE
THYROGLOBULIN

Fig. 3. Sequence relationships between the cholinesterases and homologous proteins. The bracketed regions denote the positions of intrasubunit disulfide bonds. S and H denote positions of the conserved serines and histidines.

Gene Organization

Despite their extensive diversity in structure, all AChE's in vertebrates appear to be encoded by a single gene, with alternative mRNA processing forming the basis of structural polymorphism. The evidence for these considerations is: (a) complete sequence identity of the two forms of the _Torpedo_ enzyme through Thr-535, where a sequence divergence is found[26]. The sequence divergence for the three forms of mouse AChE also occurs only in the very carboxyl-terminal end of the molecule[7]. The form containing the glycophospholipid in Torpedo contains only a unique dipeptide sequence (Ala,Cys), to which the glycophospholipid is attached to the terminal Cys. In mouse and human this region is 14 amino acids in length. The hydrophilic catalytic subunits continue for 40 residues after the divergence point; (b) RNAse digestion experiments show the single mRNA divergence in the open reading frame to be in the very region encoding the amino acid divergence; (c) a partial-length cDNA encoding the divergent sequence in the mature protein, plus a processed sequence of 24 amino acids has been isolated[24]; (d) the genomic sequence contains the alternative exons encoding the asymmetric species and hydrophobic species in Torpedo, mouse and human[27,28,30]; (e) a synthetic cDNA constructed from the appropriate exons from genomic clones, upon transfection, yields an active membrane-associated enzyme (the expressed enzyme is released upon phospholipase C treatment [cf section C]).

The components of catalytic function (Ser_{200}, His_{440} and, perhaps, an anticipated Asp or Glu) in the putative charge-relay system are found in the first exon of the open reading frame, as are the first two disulfide loops. The second exon closes the last disulfide loop, while the two alternatively spliced third exons are responsible for the differential membrane localizations.

Regulation of Acetylcholinesterase Expression

At present, little is known about the regulation of expression of this enzyme, although descriptive studies indicate it is tightly controlled by cellular excitability and intracellular Ca^{++} [31-33]. The asymmetric species does not appear until synaptogenesis ensues and action potentials are seen in muscle. Alternative exon usage involves mRNA processing from nuclear pre-RNA. Presumably, addition of the glycophospholipid occurs cotranslationally, and oligosaccharide processing and the linkage of the catalytic and structural subunits occur at the Golgi stage[33]. Little is known about factors affecting transcription, mRNA stability, or processing; cloning of the mammalian genes makes this subject appropriate for study. Other studies reveal that AChE is retained in the basal lamina after destruction of the nerve or muscle cell[34-36]. This region serves as a template for new synapse formation upon nerve regeneration.

Studies in our Laboratory

Our laboratory has been engaged in the study of AChE structure for the past 15 years, using approaches intrinsic to protein chemistry and molecular biology. These studies have yielded the first primary[12] and secondary structure[25] of the Torpedo enzyme. We then proceeded to clone the genes for mouse AChE and BuChE and provided the first published cDNA of a mammalian AChE[15]. Also, we have delineated the structure of the gene encoding the enzyme from Torpedo[30] and have established that alternative mRNA processing is responsible for the structural diversity in the AChE[28,30]. More recently, we have determined the gene structure of the murine and human AChE's for the purpose of examining comparative structures of the enzyme and regulation of gene expression[27]. The finding of a genetic mutation which may be related to the splicing mechanism has also allowed us to begin to study AChE structure in man. Our studies of AChE structure have established all of the exon-intron boundaries and alternative splice locations in both the mouse and human genes. These studies have defined the sequence of the hydrophilic, monomeric, glycophospholipid-linked and the hydrophilic oligomeric forms of mouse acetylcholinesterase.

II. BODY

The work proposed and completed in this contract largely deals with the structure of AChE and is intended to complement the studies on gene structure. One aim of the proposed research was to begin characterization of the structure of the mammalian enzymes and it immediately became evident that this would be most efficient if we used recombinant DNA-derived enzymes. Therefore, in the later phases of the contract we devoted substantial effort to this end. We are now in a position to express the mammalian enzymes at high yield from recombinant DNA sources.

A. METHODS

1. Analysis of Sequence of Torpedo 5.6S Acetylcholinesterase

Digestion of 5.6S Acetylcholinesterase with Phosphatidylinositol-Specific Phospholipase C - Purified 5.6S-AChE (20-60 mg) (1.5-2.0 mg/ml) was radio-labeled by a 1.25-molar excess of [^{3}H]diisopropyl fluorophosphate ([^{3}H]DFP) (specific activity, 10 mCi/mmol) in the presence of 0.02% sodium azide overnight at room temperature. The reaction mixture, after aging 24 h at 4°C, was dialyzed to remove unreacted DFP. The [^{3}H]-labeled enzyme was digested with phosphatidylinositol-specific phospholipase C (PI-PLC) purified from either S. aureus (20 μg/ml) or Bacillus thuringiensis (3.5 units/ml) obtained from Martin Low, Columbia University, NY. Digestion was performed in 50 mM

Tris, pH 7.2, 2 mM EDTA, 0.1% deoxycholate, 0.02% sodium azide at 37°C for 4-8 h. Digestion by PI-PLC was monitored by sedimentation on sucrose density gradients.

Fractionation of Cysteine-Containing Tryptic Peptides - The PI-PLC digest of 5.6S AChE was dialyzed against 50 mM NH_4HCO_3, pH 8.0, 0.02% sodium azide, and concentrated to ~4 mg/ml by lyophilization. The enzyme was brought to 6 M in guanidine HCl, adjusted to pH 8 with 1 M Tris base, and incubated 2 h under N_2 at 50°C with a 2-fold M excess of dithiothreitol over estimated total cysteine residues. To label cysteines, [^{14}C]iodoacetate (specific activity, 2-5 mCi/mmol) was added to the reduced, denatured enzyme in 2-fold molar excess over total thiols and allowed to react in the dark for 1-2 h at room temperature. A 10-fold excess of dithiothreitol over total thiols was added to quench unreacted iodoacetate. Following dialysis against 50 mM NH_4HCO_3, pH 8.0, the preparation was incubated with trypsin (1% w/w) at 37°C overnight and for another 2 h with additional 1% trypsin. The tryptic peptides were applied to a Sephadex G-50 column (1.5 x 200 cm), equilibrated in 50 mM NH_4OH, and eluted at a flow rate of 20 ml/h. Fractions of 3 ml were collected, monitored for absorbance at 280 nm and for ^{14}C radioactivity, and concentrated by lyophilization to 2 ml. Separation of pooled peptides was by reverse-phase HPLC on Vydac C-4 or C-18 columns in aqueous 0.1% trifluoroacetic acid using an acetonitrile gradient of 0-50% in 180 min and 50-90% in 30 min. Absorbance at 219 nm and [^{14}C]-radioactivity were monitored.

Further Purification of [^{14}C]-Labeled Tryptic Peptides - HPLC fractions containing [^{14}C]-peptides unique to the 5.6S species were pooled and further resolved on a Vydac C-4 column in 10 mM phosphate, pH 6.9, using an acetonitrile gradient of 0-50% in 150 min. Fractions containing [^{14}C]-peptides were run once more in 0.1% trifluoroacetic acid, using an acetonitrile gradient of 0-12% in 120 min. Peptides that remained unresolved after the initial HPLC fractionation were fractionated by HPLC in phosphate or on a C-18 column in trifluoroacetic acid.

Sequencing and Composition Analysis - Purified [^{14}C]-labeled tryptic peptides were sequenced by gas-phase methods, using an Applied Biosystems Protein Sequencer (Model 470A). Aliquots from sequencing steps were counted to determine the sequence position of [^{14}C]-carboxymethylated cysteinyl residues. Duplicate peptide samples were hydrolyzed in 6 N HCl at 110°C for 18 h either with prior performic acid oxidation or in the presence of thioglycolic acid alone. Amino acid analysis employed a Kontron Liquimat III amino acid analyzer with postcolumn ortho-phthalaldehyde detection. Glucosamine and ethanolamine contents were determined using an LKB 4400 amino acid analyzer with ninhydrin detection on samples hydrolyzed in vacuo in 6 N HCl at 110°C for 18 h.

Treatment with Glycopeptide N-Glycosidase - Peptide samples were dried under a nitrogen stream and redissolved in 0.1 M sodium phosphate, pH 7, 10 mM EDTA. Digestion with glycopeptide N-glycosidase (ECF 3.2.2.18; 0.2 unit/µl; Boehringer Mannheim) at 37°C for 18 h was followed by reverse-phase HPLC fractionation.

2. Antibody Production, Analysis of Specificity and In Situ Localization
Production of Site-Directed Antibodies - A hexadecapeptide corresponding to the COOH-terminal amino acids (KNQFDHYSRHESCAEL, Lys^{560}-Leu^{575}) of the catalytic subunits of the asymmetric form of acetylcholinesterase was

synthesized in the laboratory of Dr. Russell Doolittle (University of California, San Diego) by the Merrifield solid-phase method. The authenticity of the peptide was determined by gas-phase sequencing on a protein sequenc.r. The peptide was coupled to BSA by slowly adding glutaraldehyde (1 ml, 0.2%) to 2 ml of 100 mM sodium phosphate buffer (pH 7.5) containing 1.5×10^{-7} mol (10 ...g) of BSA and 7.6×10^{-6} mol of peptide. The reaction was allowed to proceed for 30 min at 22°C; then, unreacted glutaraldehyde was quenched by the addition of 0.25 ml of 1.0 M glycine. The result of the coupling reaction was evaluated by SDS-PAGE. The reduced migration of the peptide-BSA conjugate corresponded to an average incorporation of 5-10 mol of peptide per mol of BSA. In addition, the peptide-BSA conjugate was excised from the gel, and dissolved in 0.5 ml of 1.0 Tris-HCl (pH 7.0). The radioactivity incorporated into the BSA was consistent with an average incorporation of 4-5 mol of peptide per mol of BSA.

Female white New Zealand rabbits (5-6 lbs) were immunized by injection in the isolated lymph nodes of the rear leg and intradermally down the back with 0.4 ml Freund's complete adjuvant. Booster immunizations were performed intradermally after 1 mo, and 50 ml of blood was drawn 2 wk later. The serum was allowed to clot at 22°C, was clarified by centrifugation, and was frozen at -70°C in small aliquots.

CNBr-Labeled Peptides

Some of the studies on antibody reaction with the Torpedo peptides were done on CNBr derived peptides. Our CNBr treatment used standard procedures described in our earlier publications and involved dithiothreitol reduced and $[^{14}C]$-iodoacetate alkylated enzyme. The CNBr peptides were previously identified and this served as a marker for their locations. They were subsequently identified by amino-terminal sequences. Fractionation protocols involved an initial size separation on Sephadex G-50 followed by reverse phase chromatography on C-18 and C-4 columns using a 0.1% trifluoroacetic acid with the acetonitrile gradient.

SDS-Page and Western Blots - Proteins were mixed with an equal volume of
buffer containing 30 mM Tris-HCl (pH 6.8), 1.0% SDS, 5% glycerol, 10 mM DTT, 0.05 mg/ml bromphenol blue, and 0.05 mg/ml pyronin Y. Samples were boiled for 3 min, and proteins were separated by discontinuous SDS-PAGE in 1.5-mm slab gels composed of a constant ratio of acrylamide and N,N'-methylene-bisacrylamide (37:1) polymerized with ammonium persulfate (0.75 mg/ml) and N,N,N'N',-tetramethylethylenediamine (0.67 μl/ml). The stacking gel was 3.3% acrylamide in 25 mM Tris-HCl (pH 6.8), 0.2% SDS, and the separating gel was either 8% or 10% acrylamide in 75 mM Tris-HCl (pH 8.6), 0.2% SDS. The gels were run in a slab gel apparatus (model SE 500; Bio-Rad Laboratories) at 120 V constant voltage in 25 mM Tris, 190 mM glycine (pH 8.6), 0.1% SDS. Proteins were detected in the gels by staining and destaining in the presence and absence, respectively, of Coomassie brilliant blue R (0.15 mg/ml) in 50% methanol, 10% acetic acid.

Electrophoretic transfer (50 V, 150 mA, 4°C, 10-16 H) of proteins from unstained gels to nitrocellulose was performed in a transphor unit after soaking the gel in the transfer buffer (25 mM tris, 190 mM glycine, pH 8.6, 20% methanol) for 30 min. Blotted proteins were detected by staining and destaining in 50% methanol, 10% acetic acid, in the presence or absence, respectively, of Amido black (0.1 mg/ml). Immunodetection of blotted proteins was performed using a Vectastain ABC kit that uses a biotin-labeled goat anti-rabbit antibody and peroxidase-coupled avidin.

Deglycosylation of Acetylcholinesterase - The deglycosylation reactions used ^{3}H-DFP-labeled acetylcholinesterase that had been desalted on a size exclusion column and then lyophilized. [^{3}H]-DFP-labeled acetylcholinesterase (1.09 mg) was resuspended in 50 mM sodium phosphate buffer (pH 6.1) containing 50 mM EDTA, 1 mM PMSF, 10 μM pepstatin A, 0.5% NP-40, 0.5% β-mercaptoethanol, 0.1% SDS, and digested with endoglycosidase F (20 U) by incubation for 8 hr at 37°C. ^{3}H-DFP-acetylcholinesterase (1.76 mg) was deglycosylated with glycopeptidase F (1.0 U) by incubation in 1.1 ml of 250 mM sodium phosphate buffer (pH 7.4) containing 10 mM EDTA, 1.0 mM EDTA, 1.0 mM PMSF, 10 μM pepstatin A, 0.8% NP-40, 10 mM β-mercaptoethanol, and 0.5% SDS for 18 h at 37°C; [^{3}H]DFP-labeled acetylcholinesterase (2 mg) was treated for 8 h at 37°C with endoglycosidase H (1.0 U) in 0.1 M sodium citrate buffer (pH 5.5) containing 1.0 mM PMSF, 10 mM pepstatin A, 2% SDS, and 1.0% β-mercaptoethanol.

Immunoprecipitation of Acetylcholinesterase - [^{3}H]DFP-labeled acetylcholinesterase (0.4 μg) was incubated with the indicated antibodies in 200 ml of buffer A containing 20 mM sodium phosphate (pH 7.4), 150 nM NaCl, 0.02% NaN$_3$, 0.01% Tween-20, and 0.1% BSA for 2 h at 37°C. Pansorbin was washed with buffer A containing 5 mM b-mercaptoethanol and 0.5% NP-40, then washed with buffer A containing 0.05% NP-40, and finally resuspended to 2.5% (w/vol) in buffer A. Antibodies were precipitated by the addition of 800 μl of Pansorbin (15 min at 4°C). Precipitates were sedimented by centrifugation in a microfuge, the supernates aspirated, and the pellets resuspended by incubation with 200 μl of 2% SDS and 4 M urea at 90°C for 2 min. The Pansorbin was separated from the solubilized acetylcholinesterase by centrifugation in a microfuge, and the supernatant was removed for determination of radioactivity. Precipitation of [^{3}H]DFP-labeled acetylcholinesterase by the antibodies was compared to the maximal precipitation obtained by the addition of 1.0 ml of acetone rather than Pansorbin.

Preparation of Tissue for Light and Electron Microscopy - The electric organs were removed from an adult male *Torpedo* and fixed in 4% paraformaldehyde in 0.1 M PBS for 1 h at 4°C. The tissue was trimmed down to ~3 x 2-mm pieces and cryoprotected in 1.0 M sucrose with 0.5% paraformaldehyde in 0.1 M PBS for 30 min at 4°C followed by cryoprotection in 2.0 M sucrose with 0.5% paraformaldehyde in 0.1 M PBS for 1 h at 4°C. Tissue was mounted on aluminum specimen support pin so as to provide a cross-section of the electrocytes; it was then frozen in liquid propane cooled with liquid nitrogen.

Conventional electron microscopy was performed, using small pieces of electric organ that were first fixed in 2% glutaraldehyde and 2% paraformaldehyde in PBS for 1 h, followed by 1% osmium tetroxide for 1 h. After dehydration with ethanol, the tissue was embedded in EPON-Araldite resin. Ultrathin sections were counterstained with uranyl acetate and lead citrate.

Immunofluorescent Localization of Acetylcholinesterase - Thick sections (2-μm) of frozen tissue were cut on an ultracryomicrotome at -80°C and mounted on clean glass slides, using a drop of 2.0 M sucrose in a platinum loop. Sections were re-equilibrated with 0.1 M PBS for 5 min followed by 0.1 M PBS with 0.05 M glycine for 5 min. To minimize nonspecific staining, the sections were pretreated with 1% gelatin in PBS for 10 min followed by 2% normal goat sera in PBS for 10 min. Sections were incubated in specific antisera or normal rabbit or normal mouse sera for 30 min at room temperature. Dilutions of primary antibodies were 80-B (100x), 2C-9 (100x), 4F-3 (50x), CT (100x), 4E-7 (100x), and antireceptor antibodies (100x). After extensive washing in PBS, the sections were treated with either goat anti-rabbit rhodamine or goat

anti-mouse fluorescein conjugate for 20 min. The sections were again washed extensively in PBS, covered with 90% glycerol in PBS, and placed under a coverslip. Sections were examined, using a Leitz 63 x objective on a Zeiss universal microscope.

Electron Microscopic Localization of Acetylcholinesterase - Thin cryosections (0.1 nm) or thick cryosections (0.5 μm) were cut at -100°C and mounted on Formvar filmed, carbon-stabilized gold grids. The thin sections were treated much like the thick sections, except that after incubation in primary anti-sera, the sections were immunolabeled with goat anti-rabbit or goat anti-mouse IgG conjugated to either 5, 10, 15 or 30 nm gold (Janssen Life Sciences, Piscataway, NJ) for 20 min. After extensive washing in PBS, the sections were fixed in 1% glutaraldehyde/1% osmium tetroxide in PBS for 3 min. Sections were washed in distilled water and counterstained in 2% aqueous uranyl acetate for 30 min. The sections were subsequently dehydrated in ethanol and embedded in a thin film of LR white acrylic resin and polymerized as previously described[24]. Thin cryosections were viewed at 100 KeV with a JEOL 100CX electron microscope, and thick cryosections were viewed at 1 MeV with a JEM 1,000 high-voltage electron microscope.
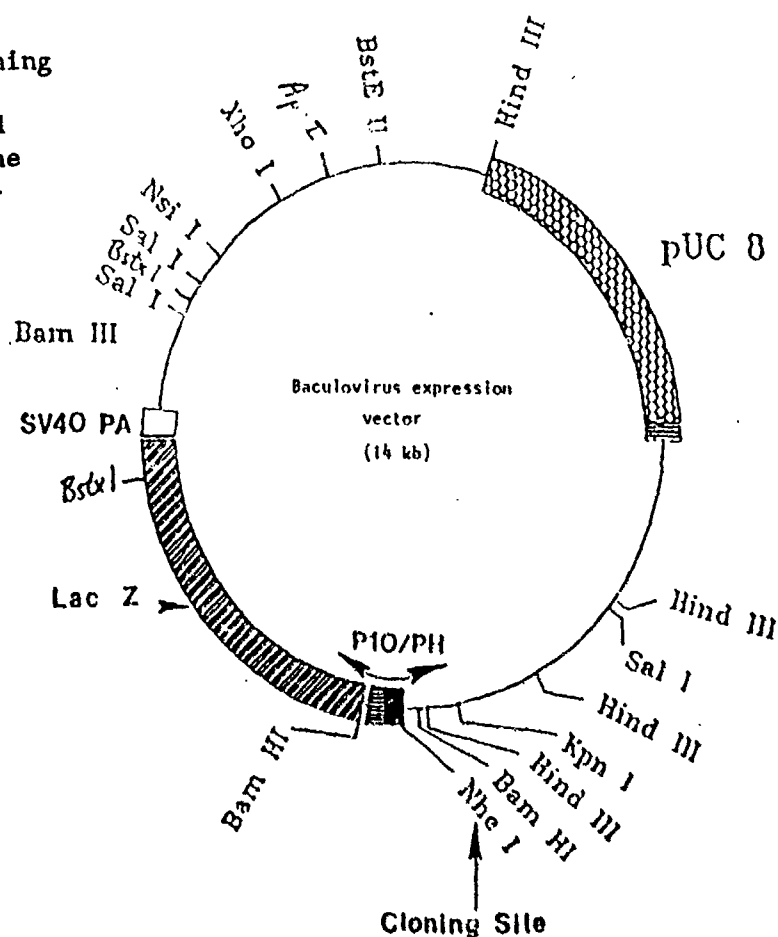
Studies of Antibody Specificity - Polyclonal antibodies and monoclonal antibodies were raised to the C-terminal and an active center peptide (KTVTIFGESAGGASVGMHILSPGSR). The monoclonal antibodies were raised in B.P. Doctor's laboratory at Walter Reed (WRAIR). Immunoprecipitation and ELISA assays employed procedures developed jointly by the two laboratories (cf 37). Similar assays were also developed to examine antigenicity of tryptic and CNBr peptides from acetylcholinesterase.

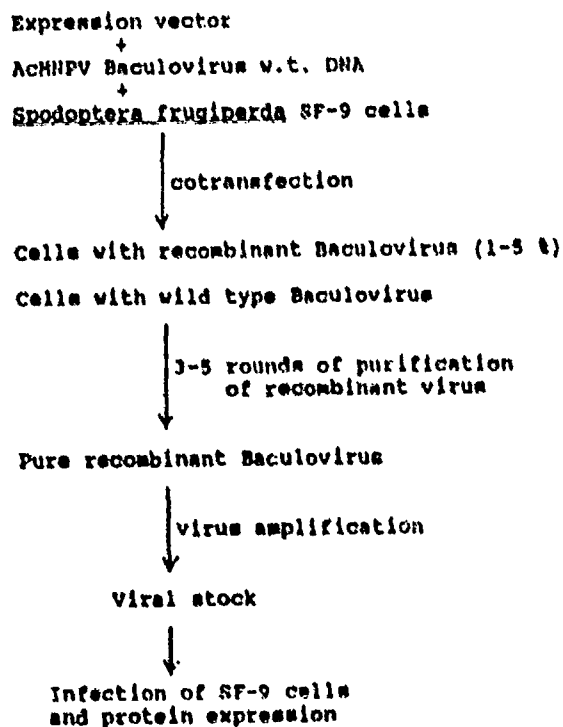Expression of Mammalian Acetylcholinesterases

To establish functional identity of the cloned cDNA or genomic DNA encoding AChE, transfection into COS-7 cells using DEAE-dextran or $Ca_3(PO4)_2$ precipitation was initially employed[38,39]. The DNA of interest (mouse cDNA or human genomic DNA) was placed in a vector behind the SV-40 early promoter. This enabled us to achieve readily detectable enzyme activity after transfection[38]. From these cells K_m and K_{ss} could be determined but insufficient enzyme was available for purification, titration of active centers and determination of V_{max}.

For the latter purpose, we have employed the Baculovirus-Spodoptera system pioneered by Summers and colleagues[40,41]. Early studies employed the standard vector and helper virus placing the cDNA behind the polyhedron promoter. More recently, we have used the dual expression system where a marker gene, β-galactosidase, is placed behind the P-10 promoter and the gene of interest behind the polyhedron promoter[42] (fig. 4). This enables us to readily detect recombinants by blue placques and saves several rounds of placque purification to obtain virus free of expression of polyhedra. Recombinant placques free of polyhedra are selected from cells grown on 24 well plates. Scale-up includes large 150 cm plates and spinner culture when the cells are grown in suspension. Fig. 5 gives the sequence of procedures employed for expression of AChE.

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Fig. 4 Expression vector for cloning Torpedo and mammalian AChE. The gene of interest is cloned behind the polyhedra promoter (PH) at the Nhe cloning site while β-galactosidase activity results from the LacZ gene placed behind the P10 promoter.

Xho I

Av I

BstE II

Hind III

Nsi I

Sal I

Bst I

Sal I

Bam III

pUC 8

SV40 PA

Bst I

Baculovirus expression vector
(14 kb)

Lac Z

P10/PH

Hind III

Sal I

Hind III

Kpn I

Hind III

Bam HI

Bam HI

Nhe I

Cloning Site

BACULOVIRUS EXPRE" O SYSTEM

Inserts:

Expression vector
+
AcHNPV Baculovirus w.t. DNA
+
Spodoptera frugiperda SF-9 cells

Torpedo AChE w.t.

Torpedo AChE E^{199}-Q

Torpedo AChE E^{199}-O

2.4 kb

BamHI — BamHI

House AChE w.t.

2.2 kb

EcoRI — Kpn I

cotransfection

Cells with recombinant Baculovirus (1-5 %)

Cells with wild type Baculovirus

3-5 rounds of purification of recombinant virus

Pure recombinant Baculovirus

virus amplification

Viral stock

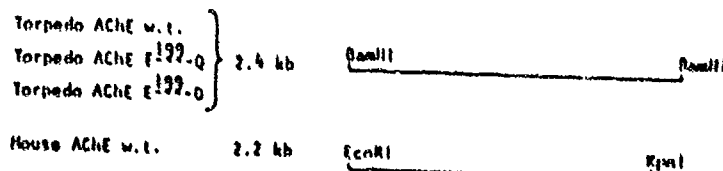Infection of SF-9 cells and protein expression

Fig. 5 Sequence of steps employed for expression of Torpedo and mouse AChE in the Spodoptera-baculovirus system.

B. RESULTS

Sequence Divergence in the Molecular Forms of Acetylcholinesterase

A unique carboxyl-terminal tryptic peptide from the hydrophobic globular (5.6S) form of *Torpedo californica* AChE that exhibits divergence in amino acid sequence from the catalytic subunit of the hydrophilic form of the enzyme was identified. The hydrophobic or amphipathic form contains an attached glycophospholipid, and the peptide could be recovered only after treatment with phosphatidy-linositol-specific phospholipase C. After reduction, carboxymethylation with [^{14}C]iodoacetate, and trypsin digestion, the resulting peptides were purified by gel filtration and high performance liquid chromatography (HPLC). The HPLC profiles of the labeled cysteine peptides from the 5.6S enzyme revealed a unique radioactive peak which was not present in digests of the asymmetric form. This radioactive peptide, which had been excluded on Sephadex G-50, eluted early as a broad peak on HPLC. The peak contained sufficient [^{14}C]-radioactivity to account for a single cysteine, but had an unusually low extinction at 219 nm for one equivalent of excluded peptide. Further HPLC purification generated multiple peaks, all of which yielded identical amino acid sequences. The difference in chromatographic behavior of the individual peaks most likely reflects heterogeneity in post-translational processing. Gas-phase sequencing and composition analysis are consistent with the sequence: Leu-Leu-Asn-Ala-Thr-Ala-Cys. The composition includes 2-3 mol each of glucosamine and ethanolamine -- which is indicative of modification by glycophospholipid. Glucosamine is also present in an asparagine-linked oligosaccharide. The two forms of AChE diverge after the Thr residue of this peptide; the peptide chain of the hydrophobic form terminates after cysteine, whereas the asymmetric form continues for 40 amino acids beyond the divergence. The locus of the divergence and absence of other sequence differences between the two forms suggest that the molecular forms of AChE arise from a single gene by alternative mRNA processing.

Antibodies to Acetylcholinesterase: Their Use in In Situ Localization

Table 1 summarizes antibodies in use for the study of the localization of AChE.

Table 1. Staining Intensity of Antibodies Used in Immunocytochemical Localization of Acetylcholinesterase in Torpedo Electric Organ

Antigen	Name	Species	Poly/Mono	Subtype	Light	EMperox[*]	EMgold[†]	Form[‡]	Reference
11 S	80A	Rabbit	Polyclonal	--	+ + +	+ + +	+ +	Both	6,8
11 S	80B	Rabbit	Polyclonal	--	+ +	+ +	+ +	Both	6,8
17 S, 5.6 S	4E7	Mouse	Monoclonal	IgG2b	+ + +	+ +	+	5.6 S	3
17 S, 5.6 S	4F3	Mouse	Monoclonal	IgG1	+ +	+	+	17 S	3
11 S	2C9	Mouse	Monoclonal	IgG1	+ + +	ND	+ + +	Both	--
11 S	2C6	Mouse	Monoclonal	IgG1	+ + +	+ +	+	ND	--
Peptide[§]	CT	Rabbit	Polyclonal	--	+ + +	+ +	+ +	17 S	--
Peptide[¶]	AS	Rabbit	Polyclonal	--	--	ND	ND	Both	--

[*] Results obtained with the electron microscope and peroxidase labeled secondary antibodies

[†] Results obtained with the electron microscope and gold labeled secondary antibodies.

[‡] Molecular form of acetylcholinesterase (5.6 S, 17 S, or both) recognized by the antibody is indicated

[§] Synthetic peptide Lys^{386}-Leu^{410}, corresponding to the COOH terminal amino acids of the catalytic subunit of the asymmetric form of acetylcholinesterase from *Torpedo californica*, was used as the antigen.

[¶] The synthetic peptide Lys^{390}-Arg^{396}, corresponding to amino acids common to both forms of acetylcholinesterase from *Torpedo californica*, was used as the antigen.

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Sequence-specific antibodies raised against a synthetic peptide corresponding to the COOH-terminal region (Lys^{560}-Leu^{575}) of the catalytic subunits hydrophilic of the hydrophilic (asymmetric) form of AChE reacted with the asymmetric form of AChE, but not with the hydrophobic (amphiphilic) form. These results confirm recent studies suggesting that the carboxyl-terminal domain of the hydrophilic form differs from that of the amphiphilic form, and represent the first demonstration of antibodies selective for the catalytic subunits of the asymmetric form. In addition, the reactive epitope to the monoclonal antibody (4E7), previously shown to be selective for the hydrophobic form of acetylcholinesterase, has been identified as an N-linked complex carbohydrate, thus defining posttranslational differences between the two forms. These two form-selective antibodies, as well as panselective polyclonal and monoclonal antibodies, were used in light- and electron-microscopic immunolocalization studies to investigate the distribution of the two forms of acetylcholinesterase in the electric organ of _Torpedo_. Both forms were localized almost exclusively to the innervated surface of the electrocytes. However, they were differentially distributed along the innervated surface. Specific asymmetric-form immunoreactivity was restricted to areas of synaptic apposition and to the invaginations of the postsynaptic membrane that form the synaptic gutters. In contrast, immunoreactivity attributable to the hydrophobic form was selectively found along the nonsynaptic surface of the nerve terminals and was not observed in the synaptic cleft or in the invaginations of the postsynaptic membrane. This differential distribution suggests that the two forms of acetylcholinesterase may play different roles in regulating the local concentration of acetylcholine in the synapse.

A large part of this study involved fluorescence microscopy and electron microscopy, which is summarized in figures 6,7 and 8. Antibodies that react with sequence common to both enzyme forms (i.e. the polyclonal antibody 80-B and the monoclonal antibody 2C-9) show staining within the postsynaptic invaginations as well as within two layers of the postsynaptic surface (fig. 6A and B). Antibodies specific for the tail subunit, monoclonal antibody 4F3, and for sequence unique to the catalytic subunit, polyclonal antibody CT, show a single layer of staining which again extends into the postsynaptic folds (fig. 6C and E). Lastly, antibody selective for the glycophospholipid-containing form of the enzyme, monoclonal antibody 4E-7, shows punctate staining on the postsynaptic membrane. Some non-specific staining can be detected on the dorsal non-inverted surface which is likely due to a common carbohydrate epitope. This becomes even more evident at high magnification (fig. 7A, B, C and D). Moreover, the punctate staining of this form of the enzyme can be contrasted for the uniform staining of the membrane seen for the acetylcholine receptor by antibody reactivity (figs. 7E and F).

The above studies with fluorescence microscopy can be carried to the electron microscopy level using colloidal gold (fig. 8A). Again, the antibodies reactive to common sequence are found on the nerve terminus and deep in the post-synaptic folds (fig. 8A). By contrast, antibody directed to the glycophospholipid containing form of the enzyme show staining only the nerve termini, which likely accounts for the punctate staining seen upon fluorescence microscopy (fig 8B). These findings point to a nerve cell body origin of the glycophospholipid form of the enzyme in the synapse. Surprisingly, this form of the enzyme is preferentially localized on the non-synaptic surface.

Analysis of Antibody Specificity and Accessibility of Epitopes on Acetyl-
cholinesterase - Polyclonal and monoclonal antibodies were generated against a
synthetic peptide (25 amino acid residues) corresponding to the sequence of
the active-site-containing region of Torpedo californica acetylcholinesterase
by coupling to bovine serum albumin or encapsulation into liposomes containing
lipid A as an adjuvant prior to immunization produced antibodies of high
titer. In order to determine whether the active-site-serine-containing region
of AChE is located on the surface of the molecule (and is, thus, accessible
for binding to antibodies) or is located in a pocket (and, thus, is not
accessible to binding), the immunoreactivity of these antibodies was determined using ELISA, immunoprecipitation, Western blots, and competition ELISA.
Both AChEs, Torpedo and fetal bovine serum, failed to react with several of
these MAbs in native form, but showed significant cross-reactivity with
denatured enzymes. Other antibodies interacted with both the native and
denatured form of the enzyme. Human serum BuChE, which has high amino acid
sequence homology to these AChEs, failed to react with the same MAbs, either
in native form or denatured form. Chymotrypsin also failed to react with
these MAbs in either form. The results suggest that the active-site-serine-
containing region of these AChEs in native state is not exposed on the surface
of the enzyme and is, most likely, located in a crevice-like conformation.

Other studies have ascertained whether the antibodies reactive with the
native and denatured form of the enzyme (as opposed to those reactive with the
native form) interact with different epitopes on this 25-amino-acid peptide.
Initially, this was pursued by cleavage of the peptide at its single
methionine and then was extended to analyzing antibody reactivity with
sequential peptides directed to this region. These studies were able to
distinguish regions of the active cluster peptide which required denaturation
and isolation to observe reactivity. This work was done in collaboration with
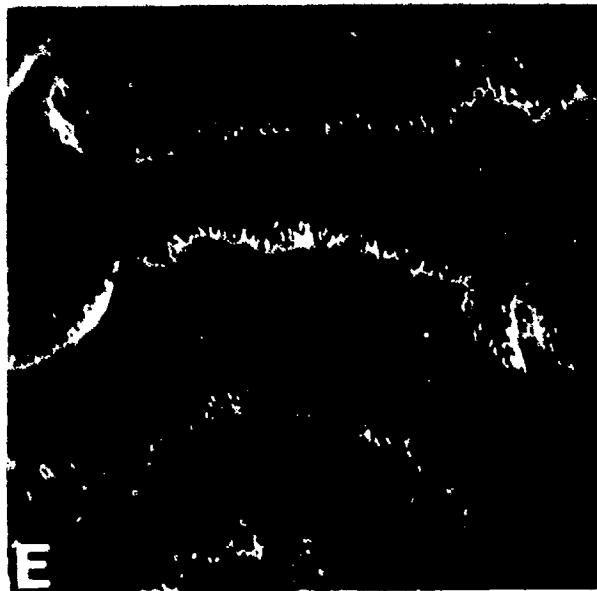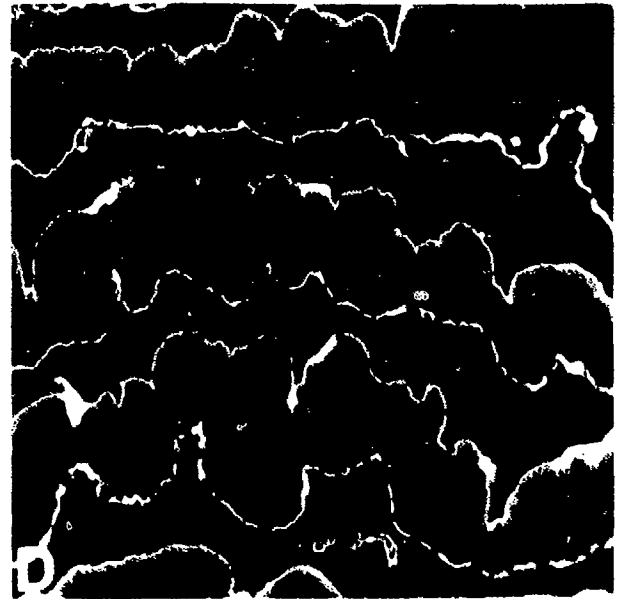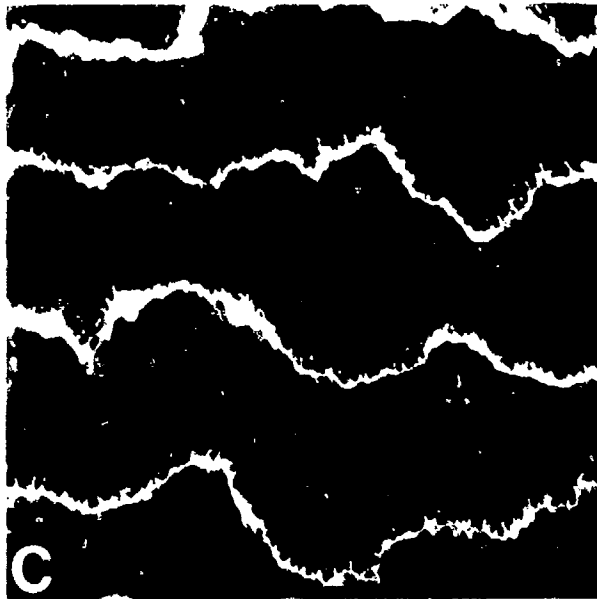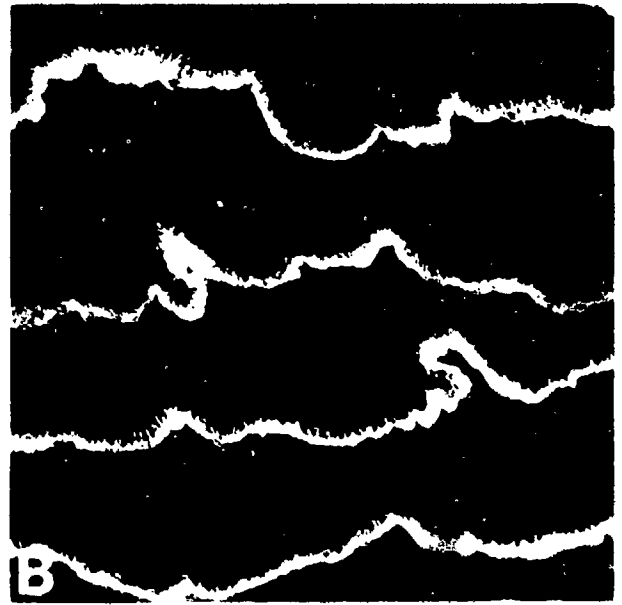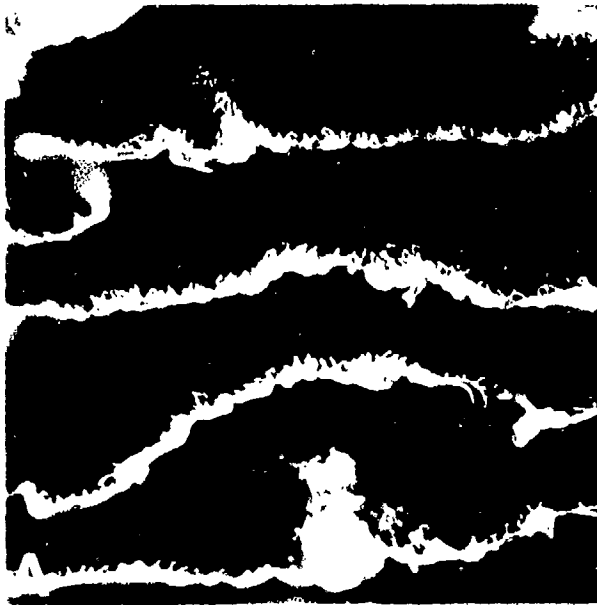the biochemistry group at WRAIR.

Two randomly selected monoclonal antibodies raised against homogeneous 11S
AChE, 4E-7 and 2C-9, which were used extensively in the immunocytochemical
studies, were characterized in terms of their sequence specificity. 4E-7
reacts with an epitope sensitive to removal of an N-linked oligosaccharide,
whereas 2C-9 reacts solely with the peptide backbone. Both have been
localized to a CNBr peptide extending between residues 44 and 83 containing
one N-linked glycosylation site. Future work was directed to localizing
further these respective epitopes. The above CNBr peptide was fractionated by
treatment with trypsin and yielded two small peptides and one large
carbohydrate containing peptide (see Table II). These three peptides were
fractionated on a C-4 reverse phase HPLC column, their identity established by
N-terminal sequencing and reactivity examined by the standard antibody assays.
As shown in Table II, 2C-9 reacts with these two tryptic peptides (residues 44
to 52 and 47 to 52) while 4E-7 reacts with the larger peptide containing the
N-linked glycosylation site (residues 53 to 83). Thus, the two important
antibodies used in the histochemistry analyses have had a rigorous epitope
definition.

Table II. Sequences of tryptic peptides found in the 44-83 amino acid peptide. The N-linked glycosylation site is marked with an asterisk.

Peptide Sequence	Antibody Reactivity
44 * 83 RFRRPEPKKPWSGVWNASTYPNNCQQYVDEQFPGFSGSEM	4E7 AND 2C9
RFRRPEPK	2C-9
PEPK	2C-9 but weak
* PWSGVWNASTYPNNCQQYVDEQFPGFSGSEM	4E-7

<u>Identification of the Active Center Surface of the Enzyme and the Sulfhydryl-Group Arrangement for Linkage to Structural Subunits</u> - DFP labeling has shown that Ser 200 is the catalytic serine. Sequences of the cholinesterases show two histidines at 425 and 440 to be conserved. Our site-specific mutagenesis work has shown that residue 440 is the serine involved in the charge-relay system (cf fig. 9), since activity is completely eliminated with the His 440 mutation.

Work is in process to identify other residues with the use of two azido phosphonates which cross-link the active site serine and a residue(s) in the active center. To date we have found a peptide starting at residue 290 cross-linked to an active-site tryptic peptide. These peptides are being characterized further.
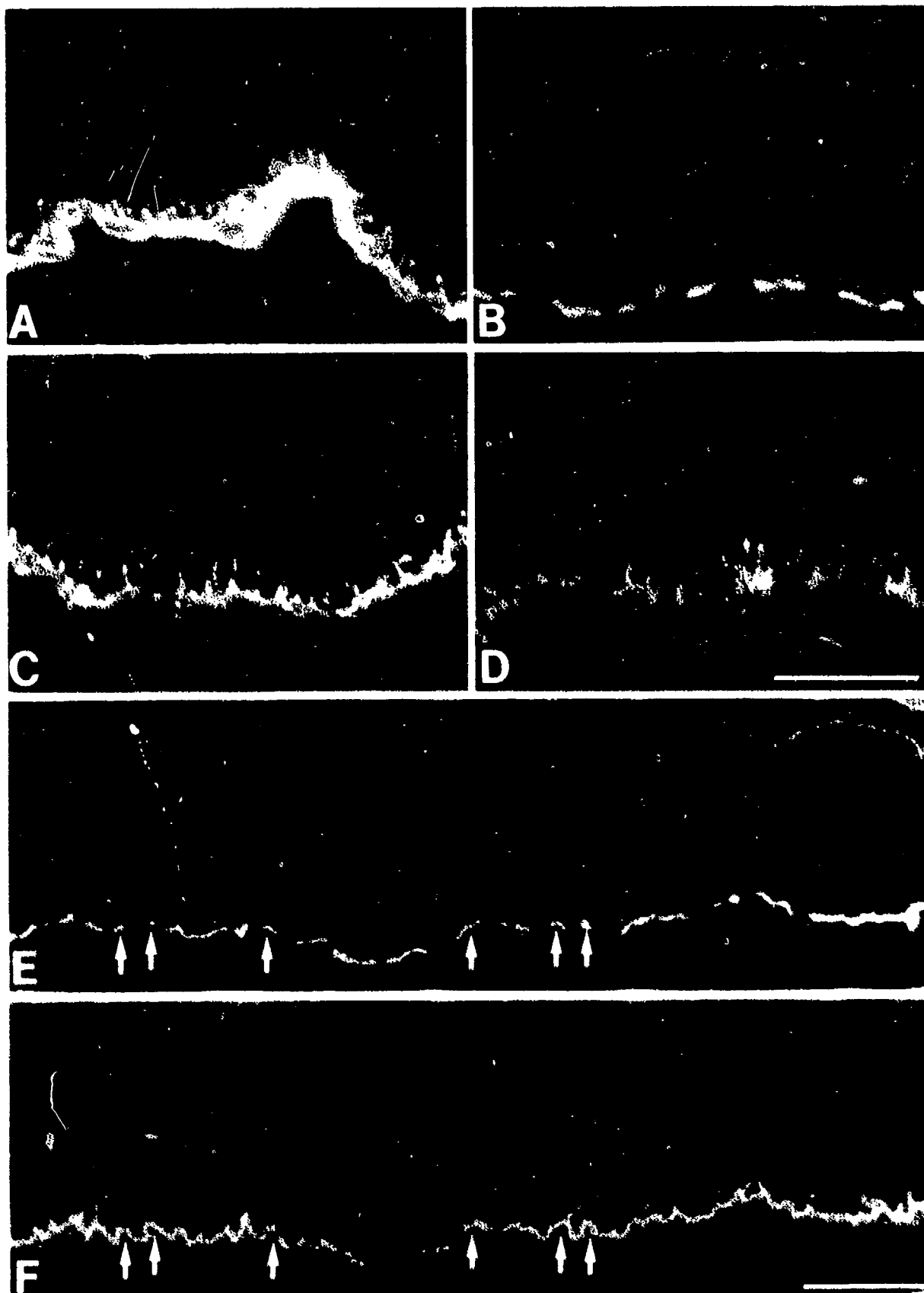
Fig. 6: Immunofluorescent localization of acetylcholinesterase in *Torpedo* electric organ. Tissue sections were prepared as described in Materials and Methods and were incubated with either (A) 80-B, (B) 2C-9, (C) 4F-3, (D) 4E-7, (E) CT, or (F) CT antibodies blocked by earlier incubation with the COOH-terminal peptide (2.8 x 10^{-7} M). These primary antibodies were visualized with rhodamine or fluorescein-labeled secondary antibodies, Bar, 10μm.

Fig. 7: High magnification of immunofluorescent staining. The primary antibodies were (A) 2C-9, (B) 4E-7, (C) 4F-3, and (D) CT. Tissue sections were also double labeled with 4E-7 (E) and antibodies selective for the nicotinic acetylcholine receptor (F). The same section is shown in both E and F, where the primary antibodies were visualized with fluorescein and Texas red-labeled secondary antibodies, respectively. The arrows (identical positions in both E and F) indicate nerve terminals stained by 4E-7 (E) that lie in shallow troughs in the postsynaptic membrane (F). Bars, 10 μm.

Fig. 8: Electron micrographs of cryosections of electric organ. (A) Tissue section showing an elongated nerve terminal cut in longitudinal section, double-labeled with 2C-9 and CT, and visualized with 5- and 10-nm colloidal gold-conjugated secondary antibodies, respectively. Arrows indicate 10-nm gold found only in the invaginations of the postsynaptic membrane and in the synaptic cleft, while arrowheads indicate 5-nm gold found in the invaginations of the postsynaptic membrane, in the synaptic cleft, and on the nonsynaptic surface of the nerve terminals. (B) Tissue section showing elongated nerve terminals cut in cross section, labeled with 4E-7, and visualized with 5-nm gold. Arrowheads indicate the 5-nm gold found selectively on the nonsynaptic surface of the nerve terminals. Bars, 1 μm.

Having completed sequencing of most of the tryptic peptides and a large number of the CNBr peptides, it was relatively easy to conduct a separate analysis of the cysteine-containing peptides in the unreduced peptide to assign intra- and intersubunit disulfide bonds. The critical relevant sequences have been published in the concluding progress report, July 1, 1987, and in references 25 and 26. These studies enabled us to show that the enzyme contains three intrasubunit disulfide loops, that Cys 231 was partially reduced, and that Cys 572 formed the intersubunit disulfide between homologous subunits. Subsequent cloning studies of mouse AChE have revealed only 7 cysteines which include the six intrasubunit and the carboxyl-terminal cysteine[15]. Since the cysteine analogous to Cys 231 is absent, the Cys at 572 or thereabouts in the mammalian enzyme must be responsible for attachment to identical catalytic subunits (homomeric associations) and attachment to the tail unit (heteromeric attachments).

Expression of Torpedo and Mammalian Acetylcholinesterases

The abundance of mammalian AChE is low and detailed study of structure-function relationships requires relatively large quantities of enzyme. With the cloning or construction of genes encoding the Torpedo, mouse and human enzymes, a valuable long-term research investment is the development of recombinant DNA expression systems. First, this would allow production of larger quantities of enzyme, and second, such a system is amenable to site-specific mutagenesis where modified enzymes can be produced. Since AChE is

Fig. 9: Secondary Structures of Torpedo Acetylcholinesterases Based on Disulfide Bond Assignments (19). The active-site serine is shown at position 200, the free cysteine at 231 and the catalytic histidine at 440. The three disulfide loops Cys 67 to 94, Cys 254 to Cys 265, Cys 402 to Cys 511, are designated as A,B,C. Cys 572 bonds in intersubunit linkages. The single arrows after 479 denotes the exon 1-2 junction while the double arrow after 535 denotes the exon-intron junction (2 to 3H or 2 to 3A) of alternative mRNA processing.
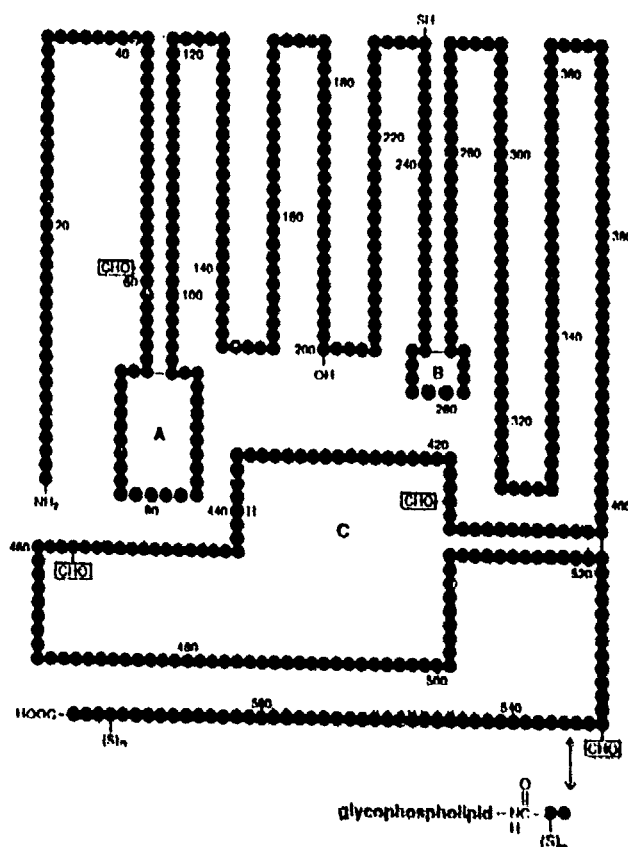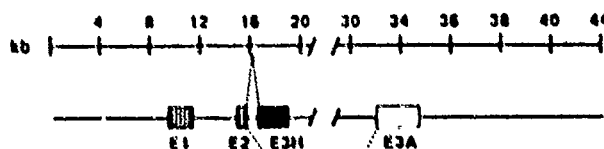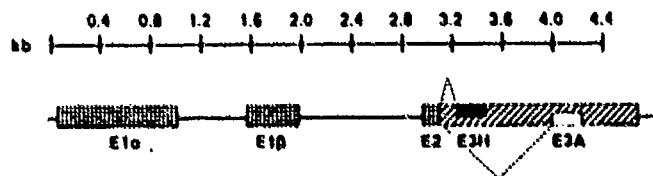

Fig. 10: Gene Structure of Torpedo and Mammalian Acetylcholinesterase. The invariant exons are shown by the dotted lines while the filled and open boxes denote alternate exons which encode a amphiphilic or a hydrophilic carboxyl-terminus. In mammalian AChE a third alternative where exon 2 is not spliced also exists and is shown by the slashed box.

glycosylated, a eukaryotic expression system is required and the one that has proven the most successful has been the baculovirus-Spodoptera system which employs a viral infection of insect cells. Cloning with the baculovirus system is a major endeavor, thus initial expression studies involved transient transfections into COS cells. Production of active enzymes with the latter system provides a confirmation that the sequence and constructions are correct. Our initial studies on the cloning of the various forms of Torpedo AChE were supported by other funds. These studies showed that the Torpedo cDNA encoding the hydrophilic form of AChE and a constructed cDNA (prepared by

loop-out mutagenesis of genomic DNA) to encode the amphiphilic (hydrophobic) catalytic subunit expressed active enzyme in COS-7 cells upon transient transfection. Moreover, the cDNA encoding the hydrophilic form yielded a substantial fraction of soluble enzyme while the cDNA encoding the amphiphilic form showed the proper processing where a glycophospholipid was attached to the carboxyl terminus[38]. This enzyme incorporated ethanolamine into its structure[38]. The synthesized enzyme resided largely on the outer face of the cell and could be released into the medium upon treatment with a phosphatidylinositol specific phospholipase C[38]. Hence, not only was active enzyme produced but the recombinant system allowed for the predicted cellular processing and cellular disposition of the biosynthesized glycoprotein. Subsequent studies showed that this system was suitable for site-specific mutagenesis and nine mutant enzymes have been studied.

This success suggested that it was prudent to develop expression systems for the mammalian enzyme in order to study the structure-function relationships outlined in this contract. Initially we expressed the cloned mouse cDNA and human genomic DNA (fig. 10) in COS-7 cells (fig. 11). Both show active hydrophilic enzyme. Of particular significance is the expression of the human enzyme since this comes directly from a genomic clone. Fortunately mammalian AChE is encoded in only 4.5-4.7 kb of gene sequence for its open reading frame. This is a convenient size for insertion into expression vectors and the COS-7 cell completes the appropriate splicing to form the correctly processed mRNA for translation.
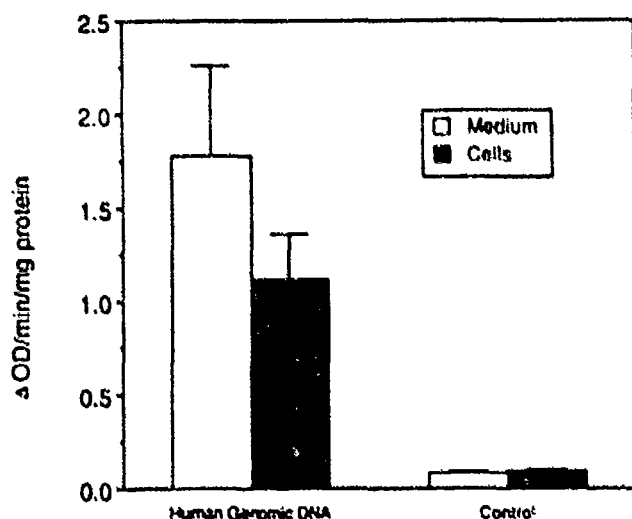

Fig. 11. Expression of human AChE in COS-7 cells. Transfection employed the and a human genomic DNA (4.7 kb) and the DEAE-dextran method. Activity is measured within the sedimented cells by the Ellman method (43).

Simultaneous with the construction of the mammalian expression system, we began studies on expression of the Torpedo gene in the baculovirus-Spodoptera cells. Initial studies achieved expression of the wild-type Torpedo amphiphilic form using the classic vector system. This entailed rather laborious studies of selection of polyhedron free cells which contained the recombinant virus. The availability of a new vector containing the β-galactosidase gene linked to the P-10 promoter facilitated our cell selection procedures and enabled us to obtain a viral stock free of polyhedra.

Encouraged by this success we immediately developed a mouse construct for expression and were able to obtain a pure viral stock of the mouse recombinant. Optimization of viral titer is shown in fig. 12. Shown below are the levels of expression of Torpedo wild-type, Torpedo mutants and mouse wild-type cDNA's (Table 3). Expression at this level results in production of ~10 mg of enzyme per liter of culture media. The increased production of soluble enzyme in the case of mouse would be expected since this is the

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hydrophilic (i.e. non-glycophospholipid) form. The data shown in Table 3 come from 24-well plates and we have been able to scale up to larger plates without loss of activity. The media used is synthetic XL-400/401 media (J.R. Scientific, Woodland, CA) to avoid contamination of AChE present in sera.
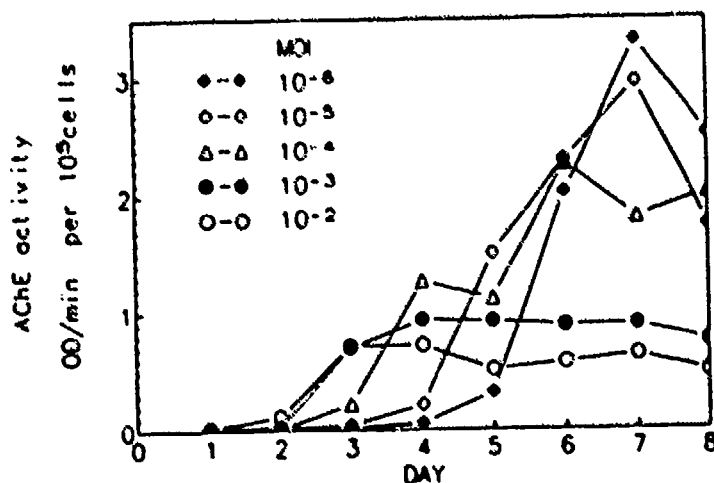
We have also grown the Spodoptera cells in spinner culture and can achieve densities of 3×10^6 cells/ml in 250 or 500 ml flasks. Use of larger flasks results in a decrease in cell density and aeration appears to be a problem. Production of AChE per cell is diminished and levels of 3-5 mg of AChE per liter are the best we have been able to accomplish in spinner culture. The lower yields may result from both aeration limitations and physical trauma to the cells.

Table 3

AChE	Viral MOI (Multiplicity of Infection)	pg AChE/cell	
		cells	medium
Torpedo w.t.	$2 \cdot 10^{-6}$	0.4	0.3
Torpedo E->Q	10^{-6}	0.3	0.2
Torpedo E->D	10^{-5}	3	2
House w.t.	$7 \cdot 10^{-6}$	0.8	2

The wild type and mutant Torpedo enzymes were expressed from cDNA's encoding the glycophospholipid-linked form of the enzyme, while the mouse cDNA encoded the form of the enzyme with hydrophilic subunits. Given are the maximal expression levels obtained in 24-well plate with $0.3 \cdot 10^6$ cells/well seven to nine days post infection. A value of 5 pg/cell is equivalent to 7 mg/liter of culture medium. Similar expression can be achieved in large surface plates or spinner culture.

Fig. 12: Optimization of viral titers for expression of Torpedo AChE. The MOI is the multiplicity of infection. Successive dilutions result in latency of activity generation, but enhanced activity. Day zero is the day of infection.

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III. CONCLUSIONS

Structural Divergence in the Acetylcholinesterase Species

Sequencing of the catalytic subunits from the asymmetric and hydrophobic forms of AChE showed that the two enzymes diverge at residue 534 and yield the sequence shown below:

Asymmetric: LLNATETIDEAERQWKTEFHRWSSYMMHWKNQFDHYSRHESCAEL
Hydrophobic: LLNATAC

The sequence of the asymmetric form was determined in studies on the previous contract. To obtain the LLNATAC sequence, we removed the acyl chains of the glycophospholipid by phospholipase C digestion. CNBr digestion yielded the deacylated peptide containing the glycan-inositol which could be isolated by size-fractionation followed by reverse phase high pressure liquid chromatography. Gas-phase sequencing yielded the above sequence. Ethanolamine and glucosamine could be detected upon amino acid analysis. This peptide was found in four peaks, all of which yielded the same peptide sequence but different posttranslational modifications.

Subsequent work with RNAse protection experiments[28] and then with genomic cloning[30] showed that the open reading frame of the enzyme is encoded by two constant exons, base -22 to 1502 and base 1503 - 1669. Base 1 is defined by the ATG start site in the leader peptide). The third exon starting at base 1670 is alternatively spliced and gives rise to two forms of acetyl-cholinesterase described above. Assignment to exon-intron boundaries shows the splice site to correspond to the appropriate position in the amino acid sequence[28,28,30]. Confirmation of the splicing mechanism has also come from loop-out mutagenesis experiments where we have been able to express the hydrophobic form of acetylcholinesterase by constructing a cDNA from the genomic clone by loop-out of the intron.

Studies of Antibody Interactions with Acetylcholinesterase

These studies have three primary directions. The first is to examine surface accessibility of various domains of the molecule. This work is being done in collaboration with B.P. Doctor WRAIR. We found that the active center of the enzyme must reside within the cleft, since monoclonal and polyclonal antibodies prepared to the active center peptide react only following denaturation. By contrast, antibodies directed to a C-terminal carboxy peptide react equally well with both the native and denatured enzyme.

The second endeavor identified epitopes for specified monoclonal antibodies. Epitopes for 2C-9 and 4E-7 have been localized to a CNBr peptide between residues 46 and 62 4E-7 reacts with the larger tryptic fragment within the region while 2C-9 reacts with a smaller tryptic peptide.

The third endeavor was to employ antibodies that distinguish the molecular forms of acetylcholinesterase in order to examine differential localization in the synapse. These studies show the asymmetric form of the enzyme to exist deep in the postsynaptic invaginations, while the hydrophobic form shows a punctate distribution, a position more removed from the postsynaptic membrane and a localization around presynaptic nerve endings.

Expression of Recombinant AChE's

This last endeavor was directed to the expression of mammalian AChE in order to develop a source of mammalian enzyme sufficient for purification and characterization. Our original studies employed transient transfection into COS cells in order to ascertain whether the cDNA or mini-gene is appropriately expressed. However, this approach does not yield sufficient AchE to titrate active centers and to characterize structurally. Accordingly, we have initiated experiments with the spodoptera-baculovirus system and can express enzyme at levels of 5-7 mgs/liter of culture medium. Wild-type Torpedo, mouse and human AChE's have been expressed and it has been possible to express the enzyme from both cDNA and genomic inserts. This system is amenable to making mutants and thus a large number of mutant AChE's should emerge in subsequent studies.

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